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(54) **Hybridoma and production of biologically active substance.**

(57) Disclosed are (1) a hybridoma carrying a vector for expressing a fibroblast growth factor (FGF) protein gene; (2) a method for producing the hybridoma of (1) which comprises transforming a hybridoma with the vector for expressing the FGF protein gene; and (3) a method for producing a biologically active substance which comprises cultivating in a culture medium the hybridoma obtained by the method of (2) using a hybridoma producing a biologically active substance other than the FGF protein, producing the FGF protein and producing and accumulating the biologically active substance in a culture, and recovering the biologically active substance, whereby the biologically active substance can be efficiently produced and recovered using the serum-free medium, which is advantageous for industrial production and very useful for an improvement in the breeding of the hybridoma.

EP 0 459 372 A3

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EP 91 10 8635
Page 2

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)

			TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
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CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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EP 0 459 372 A2

culture methods using serum-free culture media have been established yet.

The present inventors considered that such problems could be solved if a mutant strain exhibiting high proliferative activity, even in serum-free culture media, could be obtained. Several attempts were made to improve the cells proliferative activity by conventional methods such as mutations. However, satisfactory results were not obtained. Even if a desired mutant strain is obtained, that method is time-consuming and cannot be said to be a sure method.

In recent years, expression vectors using animal cells as host cells have been developed. The present invention was completed by improving proliferative activity of hybridomas using genetic engineering techniques, thus solving the above-mentioned problems.

BRIEF DESCRIPTION OF DRAWING

Fig. 1 shows the time course of an agitation culture of a bFGF gene-free original cell line (Fig. 1(A)) and a bFGF gene transfected cell line (Fig. 1(B)) at a low cell density (refer to Example 4).

DISCLOSURE OF INVENTION

The present inventors have diligently studied and established a method for cultivating cells from a single hybridoma cell or a low cell density, using serum-free culture media. More specifically, the present inventors have discovered that cell proliferation potency is significantly improved by introducing FGF genes into the cells.

In accordance with the present invention, there is provided (1) a hybridoma carrying a vector for expressing a fibroblast growth factor (FGF) protein gene, (2) a method for producing the hybridoma of the above item (1), which comprises transfecting a hybridoma with the vector for expressing the FGF protein gene, and (3) a method for producing a biologically active substance, which comprises cultivating in a culture medium the hybridoma obtained by the method of the above item (2) using a hybridoma producing a biologically active substance other than a FGF protein, producing the FGF protein and accumulating the biologically active substance in a culture, and recovering the biologically active substance.

BEST MODE FOR CARRYING OUT THE INVENTION

In the present invention, any FGF protein may be used as long as it is a polypeptide or a protein having FGF activity. Further, as the FGF protein, either bFGF protein, which is basic in its isoelectric point, or aFGF which is acidic in its isoelectric point may be used.

Furthermore, the FGF proteins may contain FGFs which are known to be obtained by recombinant DNA technology [PCT International Publication No. WO/87/01728; FEBS Letters 213, 189 (1987); and EP Publication No. 237,966 (Japanese Patent Unexamined Publication No. 63-226287/1988)] and FGF muteins [EP Publication No. 281,822 (Japanese Patent Unexamined Publication No. 2-193/1990); Biochemical and Biophysical Research Communications 151, 701 (1988); and EP Publication No. 326,907] the disclosures of which are hereby incorporated by reference.

The above-mentioned FGF muteins are obtained essentially by variations of the amino acid sequences of the original peptides or proteins. Such variations include addition of amino acid residue(s), deletion of constituent amino acid residue(s) and/or substitution of constituent amino acid residue(s) by different amino acid residue(s).

Such addition of amino acid residue(s) includes addition of at least one amino acid residue.

Such deletion of constituent amino acid residue(s) includes deletion of at least one FGF-constituent amino acid residue.

Such substitution of constituent amino acid residue(s) by different amino acid residue(s) includes substitution of at least one FGF-constituent amino acid residue by at least one different amino acid residue.

At least one amino acid residue in the mutein which has at least one amino acid residue added to the FGF excludes methionine derived from an initiation codon used for peptide expression and a signal peptide.

The number of the added amino acid residue(s) is at least one. However, it may be any number as long as FGF characteristics are not lost. More preferably, some or all of the amino acid sequences of proteins which have homology with the FGFs and which exhibit activity similar to that of the FGFs are included.

As to the number of the deleted FGF-constituent amino acid residue(s) in the mutein which lacks at least one FGF-constituent amino acid residue, it may be any number as long as FGF characteristics are not lost.

Examples of the deleted constituent amino acid residues include the 10 residues on the amino terminal

The FGF muteins may be obtained by a combination of two or three of the above-mentioned addition, deletion and substitution.

As the FGF mutein, a mutein is preferable in which at least one human bFGF-constituent amino acid residue is substituted by at least one different amino acid residue.

Any FGF protein gene may be used in the present invention as long as it codes for the above-mentioned proteins. For example, such FGF protein genes can be obtained from the cells of the brains, the retinas, the kidneys and the prostates of mammals such as humans, monkeys, rabbits, sheep, bovines, chickens, dogs, pigs and mice, or from various FGF protein-producing transformant cells. The FGF protein genes can be obtained by methods known in the art, for example, by using the cloning technique of extracting messenger RNA (mRNA) from the above-mentioned cells, preparing its complementary DNA (cDNA) enzymatically, binding a suitable vector thereto followed by proliferation in host cells such as E. coli, and selecting a single colony having the vector into which the FGF protein gene is incorporated. Further, various commercially available cDNA libraries may also be used such as bovine brain cDNA, bovine retina cDNA, chicken brain cDNA, dog kidney cDNA and human brain cDNA. Furthermore, the amino acid sequences of bovine or human bFGF and aFGF and the nucleotide sequences of their genes have already been published [T. Kurokawa et al., FEBS Letters 213, 189-194 (1987); and F. Esch et al., Biochem. Biophys. Res. Commun. 133, 554-562 (1985)]. Based thereon, therefore, oligonucleotides having appropriate nucleotide sequences may be prepared by organic synthesis according to methods known in the art, and can be used as probes for colony selection in the above-mentioned cloning. Additionally, the FGF protein genes can be prepared from vectors (for example, plasmids) into which the FGF protein genes are incorporated, or from transformant cells carrying the vectors. The FGF protein genes can further be prepared by chemical synthesis, based on the known amino acid sequences of aFGF and bFGF or the muteins thereof, or based on the nucleotide sequences of the genes thereof. In the present invention, not only the genes coding for bFGF or aFGF, but also mutant genes thereof (for example, genes coding for FGF muteins) can be used as the FGF protein genes as long as FGF activity is not lost when the genes are expressed as the FGF proteins.

The FGF protein genes are introduced into the hybridomas in the constitutively or inductively expressible state. For this purpose, it is preferable to introduce the appropriate vector for expressing the FGF protein gene (hereinafter also briefly referred to as "expression vector") into the hybridoma to transform it. The expression vector is incorporated with a DNA sequence in which a constitutively or inductively operable promoter, a translation initiating codon (ATG) and the FGF protein gene are arranged in this order.

Examples of vectors used for the expression vectors include but are not limited to pSVL [Mol. Cell. Biol. 4, 817 (1984)], pCH 110 [J. Mol. App. Genet. 2, 101 (1983); and Cell 39, 653 (1984)], pKSV-10 [Cell 23, 175 (1981)], pSV2 [Proc. Natl. Acad. Sci., U.S.A. 78, 2072 (1981)], $pBTV_{69T}$ [Methods in Enzymol. 101, 387 (1983)], pHEBo [Mol. Cell. Biol. 5, 410 (1985)], pZIP-NeoSV [Cell. 37, 1053 (1984)] and $pMAM_{neo}$ [Nature 294, 228 (1981)] the disclosures of which are hereby incorporated by reference.

Any promoter may be incorporated upstream from the above-mentioned translation initiating codon as long as it is suitable for the hybridoma used for expression of the FGF protein gene. Examples of such promoters include the promoter of a metallothionein gene [D. H. Hamer, Ann. Rev. Biochem. 55, 913 (1986)], and promoters existing in the promoter region of SV (simian virus) 40 [Okayama et al., [Mol. Cell. Biol. 3, 280-289 (1983)] and various retrovirus LTR (long terminal repeat) regions the disclosures of which are hereby incorporated by reference.

Examples of retrovirus LTR region-derived promoters include Abelson murine leukemia virus (A-MuLV) [S. P. Goff et al., Cell 22, 777-785 (1980)], Moloney mouse leukemia virus (M-MuLV) [Niwa et al., Cell 32, 1105-1113 (1983)], adult T cell leukemia virus (ATLV) [Yoshida et al., Proc. Natl. Acad. Sci. U.S.A. 79, 6899-6902 (1982)], and avian sarcoma virus (ASV) [Kitamura et al., Nature 297, 205-208 (1982)] the disclosures of which are hereby incorporated by reference.

The promoter of the metallothionein gene is also constitutively expressed, but the expression is induced more strongly by heavy metals such as Cd, Zn, Hg, Ag, Cu and Au.

In the present invention, one or more of the above-mentioned promoters may be used.

Further, upstream from the 5'-terminus of the FGF protein gene, the above-mentioned expression vector may have a nucleotide sequence coding for, for example, the signal peptide consisting of the -21st to -1st codons shown in Fig. 2 of Japanese Patent Unexamined Publication No. 61-52293/1986 the disclosure of which is hereby incorporated by reference.

Furthermore, the above-mentioned expression vector may have TAA, TGA or TAG as a translation terminating codon at the 3'-terminus of the FGF protein gene. In particular, TAG is preferable.

The expression vectors used for transfection of the hybridomas may further have enhancers. Such

or a filter. Biologically active substances other than the FGF protein, which are produced by cultivation of the hybridomas, are recovered from a supernatant obtained by filtration or centrifugation, when the substances are accumulated in the culture supernatant. When the substances are accumulated in the cells, the cells obtained by filtration or centrifugation are treated by physical methods (for example, ultrasonic oscillation, French press and Dyno-Mill) or chemical methods (for example, guanidine hydrochloride) to extract the products, thereby obtaining a supernatant.

The biologically active substances can be separated from the above-mentioned supernatant and purified by appropriate combinations of conventional separating and purifying methods. For example, when the biologically active substances are proteins or peptides, such methods include methods utilizing a difference in solubility such as salt precipitation and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion-exchange chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in hydrophobicity such as reverse-phase high performance liquid chromatography and methods utilizing a difference in isoelectric point such as isoelectric point electrophoresis.

Hybridomas can be cultivated in serum-free media even under low inoculum density by introducing the FGF protein genes into human-human hybridomas and expressing them. Further, cell proliferation is enhanced by the expression of FGF proteins, whereby the amounts of biologically active substances are also increased. Accordingly, biologically active substances such as antibodies can be efficiently produced and recovered using serum-free media. This is particularly advantageous for industrial production.

Moreover, cloning in serum-free media becomes possible by introduction and expression of the FGF protein genes. This means that a single cell can be selected in a serum-free medium, which is very useful for an improvement in the reproduction of the hybridomas.

The present invention will hereinafter be described in more detail with the following Examples. It is understood of course that these Examples are not intended to limit the scope of the invention. Transfected human-human hybridoma HPO-75.29-H74 obtained in Example 2 described below was deposited with the Institute for Fermentation, Osaka, Japan (IFO), under the accession number IFO 50245 on May 8, 1990. This hybridoma cell line was deposited with the Fermentation Research Institute, the Agency of Industrial Science and Technology, the Ministry of International Trade and Industry, Japan (FRI), under the accession number FERM BP-2939 on June 1, 1990 under the Budapest Treaty.

Example 1

(1) Introduction of bFGF Gene into Human-Human Hybridoma

As a vector containing bFGF gene, pTB732 [R. Sasada et al., Mol. Cell. Biol, 8, 588-594 (1988)] was used. Antibody high-productive cell line HPO-75.29C derived from anti-HBsAg human MoAb-producing human-human hybridoma HBW-4.16 [K. Harada et al., Bio/Technology 7, 374-377 (1989)] was suspended in a 0.3 M sucrose solution (sterility) at a density of $1X10^7$ cells/ml. In 100 µl of the resulting suspension, 25 µg of plasmid pTB732 treated with restriction enzyme ClaI and 5 µg of plasmid $pRSV_{neo}$ [Science 221, 551 (1983)] treated with restriction enzyme BamHI were mixed. The resulting mixed solution was introduced into a centrifuge chamber of an electroporation device (DPE), and centrifuged at 2,000 rpm for 2 minutes. Then, the voltage of 1,000 V/3 mm was once applied thereto for 30 seconds, followed by standing for 10 minutes in ice water. The cells subjected to electroporation treatment were suspended at a density of $1X10^5$ cells/ml in IsF medium containing antibiotic G418 (Sigma, U.S.A.) at a concentration of 1 mg/ml, and inoculated into each well of 96-well multiplate in an amount of 100 µl/well, followed by cultivation in a carbon dioxide incubator at 37°C for 2 weeks. Then, 7 proliferated clone cell lines were selected.

(2) Detection of Introduced Gene

The cells ($1X10^9$ cells) were suspended in 200 µl of 10 mM Tris-HCl buffer (pH 7.4), and the same amount of 0.4 M Tris-HCl buffer (pH 8.0), 100 mM EDTA, 1% SDS and 200 µg/ml protease K (BRL, U.S.A.) was added thereto, followed by heat treatment at 60°C for 1 hour. The same amount of phenol-chloroform was added thereto to take a supernatant, and the same amount of chloroform was further added to take a supernatant again. To the resulting DNA solution, 0.1 part by volume of 3 M NaOH was added, and heat treatment at 60°C was conducted for 1 hour, followed by cooling to room temperature. Then, the same amount of 2 M ammonium acetate was added thereto for neutralization. The solution was adsorbed by a nitrocellulose filter previously wetted with 1 M ammonium acetate by using a dot blotting device (Bio RAD).

Table 1
Comparison of Original Cell Lines and bFGF Gene-Introduced Cell Lines in Proliferated Amounts (Plate Cultivation)

Cell line	Cell number ($X10^3$/ml) Cultivation for 7 days	Cultivation for 14 days
HPO-75.29C	2.2	15
HPO-75.29-N27	7.7	170
HPO-75.29-N58	7.7	190
HPO-75.29-N73	12.0	230
HPO-75.29-H54	12.5	210
HPO-75.29-H74	17.0	330

As can be seen from Table 1, the original Cell Line HPO-75.29C was low in proliferated amounts biginning with an inoculation of $1X10^3$ cells/ml. However, for the bFGF gene-introduced cell lines, a significant enhancement in proliferation was observed.

Example 4

Each of human-human hybridoma HPO-75.29-H74 (B) cell line (IFO 50245, FERM BP-2939) most proliferated in Example 3 and original cell line human-human hybridoma HPO-75.29C (A) was inoculated into serum-free medium PEG-86-1 at a density of about $1X10^3$/ml, and an agitation culture was carried out at 37°C for 15 days using 125-ml Techne spinner flasks. As a result, the results shown in Fig. 1 was obtained. Referring to Fig. 1, -●- indicates the total number of cells, -o- indicates the number of viable cells, and -▲- indicates the production amount (µg/ml) of anti-HBsAg human MoAb.

As apparent from Fig. 1, the bFGF gene-introduced cell lines remarkably increased in cell proliferation and antibody production, compared to the original cell line.

Example 5

Each of human-human hybridoma HPO-75.29-H74 and the original cell line, human-human hybridoma HPO-75.29C, was inoculated into 100 µl of serum-free medium PEG-86-1 in each well of 96-well plastic plates so as to contain 0.5 cell/well, and incubated in a 5% carbon dioxide incubator at 37°C for 15 days. For the original cell line HPO-75.29C, no cell proliferation was observed in all of the wells of the four 96-well plates, namely in 384 wells. For HPO-75.29-H74 cell line, cell proliferation was observed in 10 wells of 384 wells.

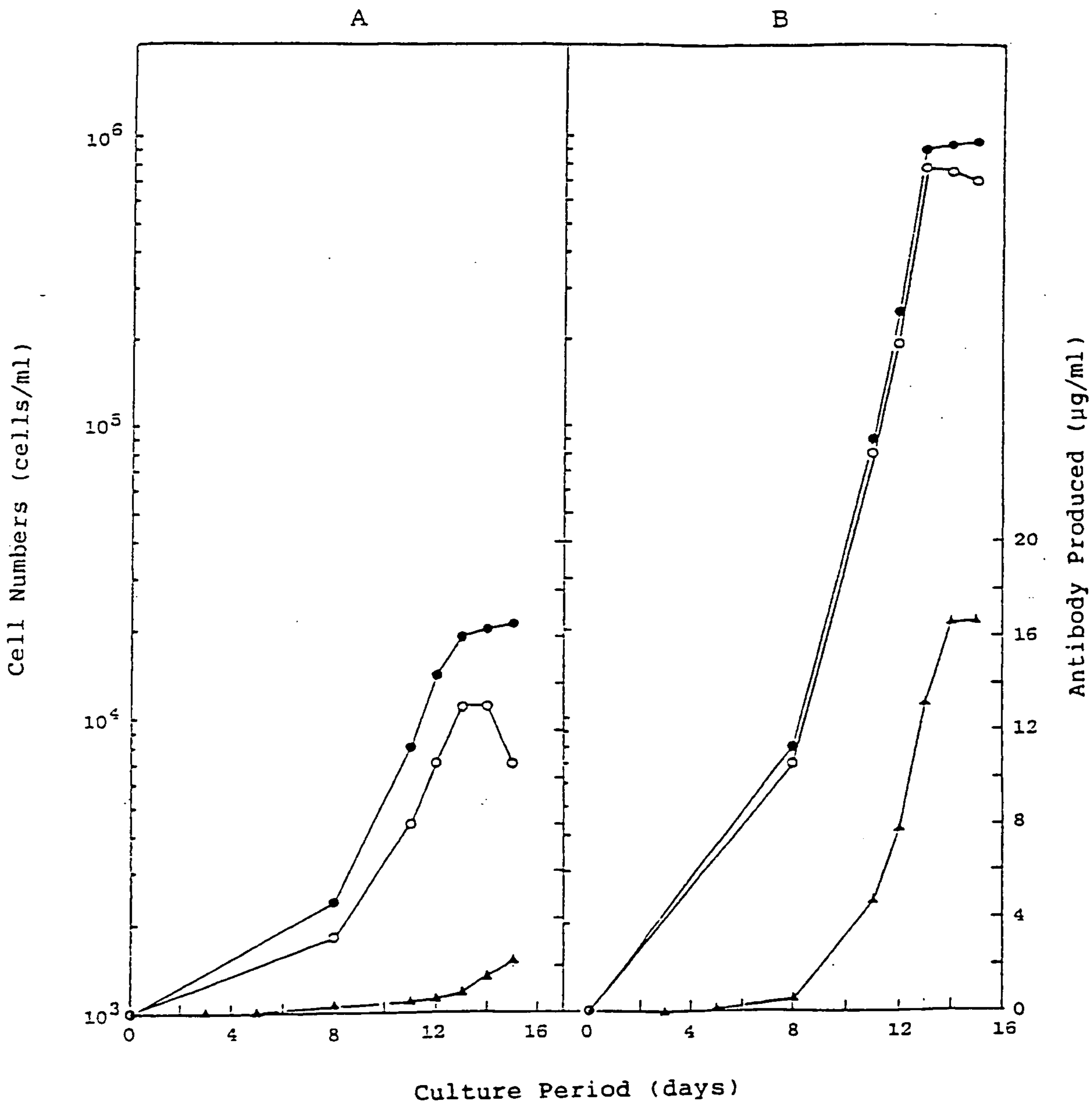
Claims

1. A hybridoma carrying a vector for expressing a fibroblast growth factor (FGF) protein gene.

2. A hybridoma in accordance with claim 1, which produces biologically active substance other than a FGF protein.

3. The hybridoma in accordance with claim 2, in which the biologically active substance is a monoclonal

Fig. 1


A
B
Cell Numbers (cells/ml)
10^6
10^5
10^4
10^3
Antibody Produced (μg/ml)
20
16
12
8
4
0
0
4
8
12
16
Culture Period (days)